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Recombinant Swinepox Virus Expressing β-Galactosidase: Investigation of Viral Host Range and Gene Expression Levels in Cell Culture

Juan Bárcena and Rafael Blasco[1]

Centro de Investigación en Sanidad Animal–I.N.I.A., Valdeolmos (Madrid) E-28130, Spain

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Swinepox virus (SPV) has been proposed as a potential vector for generating recombinant vaccines for swine. However, little is known about important aspects of SPV biology, such as the functionality of SPV promoters or the host range of SPV. Using a transient expression assay, well-characterized vaccinia virus promoters were shown to be active in cells infected with SPV. A recombinant SPV expressing β-galactosidase (β-gal) was constructed and characterized. The *E. coli LacZ* gene was placed under the control of a strong vaccinia synthetic early/late promoter and was inserted by homologous recombination in a noncoding region of the SPV genome. The recombinant SPV expressing β-gal was used to characterize the host range of the virus by measuring protein expression and virus production in different cell lines. In general, SPV expressed more protein and grew more efficiently than vaccinia virus in porcine cell lines. Surprisingly, the recombinant SPV was able to infect and replicate in several cell lines of nonswine origin. The virus directed regulated early and late gene expression of β-gal in those cells and formed blue plaques in cell monolayers in the presence of X-gal. Upon infection with the recombinant SPV, there was a significant level of viral replication, and the virus can be serially passaged in some nonswine cell lines. The data presented suggest that despite the strict host tropism of SPV, the virus exhibits a relatively broad host range in cell culture.

Key Words: Poxviridae; swinepox virus; eukaryotic expression vector; *E. coli* β-galactosidase.

INTRODUCTION

Poxviridae are large DNA containing viruses that characteristically replicate in the cytoplasm of the host cell, where both DNA replication and RNA transcription take place. The coding of many enzymes involved in virus replication and gene expression makes the poxviruses relatively independent of the corresponding cellular functions. The best studied poxvirus, vaccinia virus (VV), has a wide host range, being able to infect many different mammalian species. Also, VV readily infects cell lines derived from widely different species. VV host range is modulated by at least five genes: four encoded by VV or rabbitpox virus and a third encoded by the closely related cowpox virus (Beattie *et al.,* 1996; Brooks *et al.,* 1995; Chang *et al.,* 1995; Drillien *et al.,* 1981, 1978; Gillard *et al.,* 1985; Perkus *et al.,* 1990).

Poxviruses have a potential as vectors for immunization against human and veterinary diseases. One important characteristic of vectors, in particular for animal vaccines, is the host restriction of the viral vector itself. Ideally, a recombinant veterinary vaccine should replicate to some extent in the target animal, in order to increase the potency, and be unable to be transmitted to other species. Thus, host restriction of poxviruses with potential as vectors is of importance in the development of safe and potent vaccine vectors.

Swinepox virus (SPV) is the only member of the genus *Suipoxvirus,* belonging to the family Poxviridae. SPV's natural host is the pig, where it causes a usually mild, generalized infection (for review, see (Mahnel, 1989)). The natural characteristics of SPV infection make it well suited for the development of recombinant vaccines, and its use to vaccinate against other diseases has been considered (Tuboly *et al.,* 1993; van der Leek *et al.,* 1994). However, little is known regarding the biology of SPV, and more information is needed to optimally address the critical issues regarding vaccine vector safety and immunogenicity. SPV can be grown in porcine primary cell cultures and can be adapted to pig cell lines. SPV has been experimentally inoculated in different species and displays a narrow host range *in vivo* (Datt, 1964; Schwarte and Biester, 1941). In addition, the virus has been reported to infect only cells of swine origin (Garg and Meyer, 1972; Kasza *et al.,* 1960).

In this work we report the isolation and characterization of a recombinant SPV expressing β-gal and show a detailed study of the behavior of the virus in cell cultures of porcine and nonporcine origin.

RESULTS

Transient expression of β-gal

Because there is no functional information available regarding SPV promoter sequences, we initially tested

[1] To whom correspondence and reprint requests should be addressed at CISA-INIA, E-28130 Valdeolmos (Madrid), Spain. Fax: 34-1-620 22 47. E-mail: blasco@inia.es.

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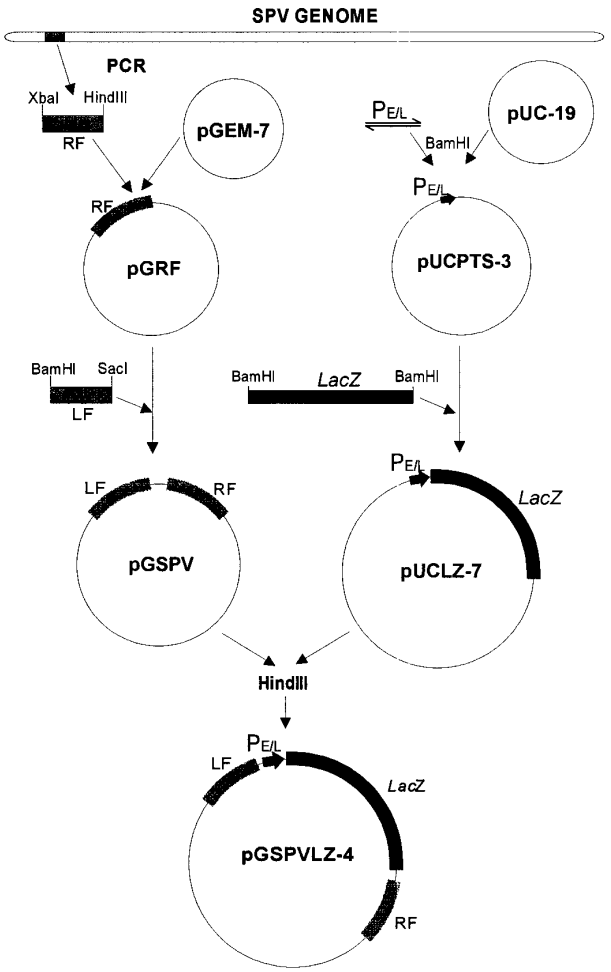


FIG. 1 Design of a SPV insertion vector. Two SPV DNA segments (LF and RF) close to the left end of the genome were amplified by PCR and inserted into plasmid pGEM-7 to generate pGSPV. To construct the $P_{E/L}$-*LacZ* cassette, the promoter sequence was first generated by annealing two synthetic oligonucleotides and was cloned in plasmid pUC-19. Subsequently, the coding sequence of the *LacZ* gene was inserted downstream of the promoter to create pUCLZ-7. Finally, plasmid pGSPVLZ-4 was generated by inserting the $P_{E/L}$-*LacZ* cassette between the SPV sequences in pGSPV. LF, left flanking sequence; RF, right flanking sequence.

our *LacZ* cassettes in a transient expression assay to determine whether VV promoters are functional in SPV-infected cells. Two plasmids were used in these assays: plasmid pSC11 (Chakrabarti *et al.,* 1985), in which the *LacZ* gene is under the control of the late VV promoter P_{11}, and plasmid pUCLZ-7 (see Fig. 1), which carries the *LacZ* gene under the control of a synthetic VV early/late promoter, $P_{E/L}$. Both plasmids were tested for their ability to direct expression of β-gal in SPV- or VV-infected PK-15 cells. As expected, β-gal expression was dependent on the presence of viral transcriptional machinery, since no β-gal expression was detected after transfection of either plasmid in uninfected cells (data not shown). Similar levels of β-gal were detected in lysates from either SPV or VV infected cells (Fig. 2), indicating good cross-recognition of VV promoters by the SPV RNA polymerase. Levels of β-gal in cells transfected with pUCLZ-7 were about 50% higher than those obtained with pSC11. Given that SPV efficiently recognized VV $P_{E/L}$ promoter, we selected this promoter for the development of a SPV cloning and expression system.

Design of a SPV virus insertion/expression system

A cloning system that would allow the construction of SPV recombinants was developed. The system was designed to mediate insertion of foreign genes into the SPV genome by homologous recombination. We constructed plasmid pGSPV-LZ4, containing SPV flanking sequences, the $P_{E/L}$ promoter, and the *LacZ* gene (Fig. 1). This plasmid was devised to direct the insertion of the $P_{E/L}$-*LacZ* cassette into a 367-nucleotide-long intergenic region located between ORFs C18L and C19L in the left terminal region of the SPV genome (Massung *et al.,* 1993). Foreign genes can be inserted in pGSPV-LZ4 in place of the LacZ gene by using a *Bam*HI site located downstream of the promoter.

Isolation of a recombinant SPV expressing β-gal

Similar to systems developed for the construction of other recombinant poxviruses, infection/transfection was used to allow insertion of the foreign gene by homologous recombination. Plasmid pGSPV-LZ4 was transfected into PK-15 cells infected with SPV. After 6–7 days, dilutions of the progeny virus were used to infect fresh monolayers of ESK-4 cells, which were stained with X-gal to detect β-gal expression. Blue, β-gal-expressing virus plaques appeared at an approximate frequency of 0.05%

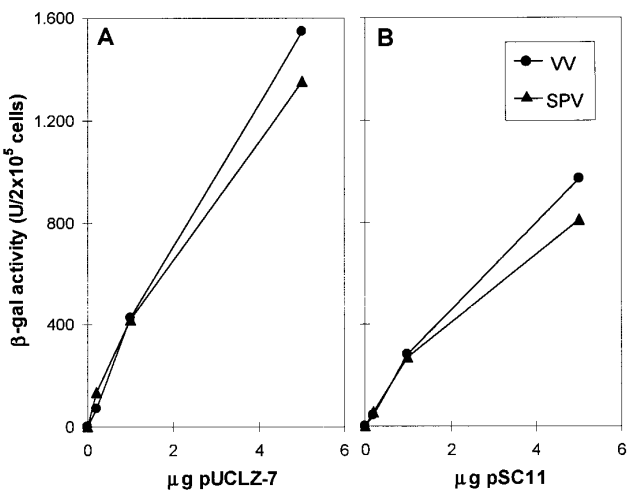


FIG. 2. Transient expression of β-gal. PK-15 cells infected with VV (●) or SPV (▲) at a m.o.i. of 5 PFU per cell were transfected with different amounts of plasmid pUCLZ-7 with β-gal under control of a synthetic E/L promoter (A) or a plasmid (pSC11) with β-gal under control of a late promoter (B). After 4 days, the cells were harvested and assayed for β-galactosidase activity (expressed as nmoles of ONPG hydrolyzed in 1 min at 37°C).

TABLE 1

β-Galactosidase Activity in Different Cell Lines Infected with SPV-LZ4 or VV-LZ2

		β-Galactosidase activity[a]			
		VV-LZ2		SPV-LZ4	
Cell line	Origin	+ AraC[b]	−AraC	+AraC	−AraC
PK-15	Porcine	55 ± 2.6	1,429 ± 39	112 ± 14.6	2,120 ± 92
ESK-4	Porcine	18 ± 0.1	250 ± 3	74 ± 3.0	1,727 ± 45
ST	Porcine	16 ± 0.6	141 ± 3	112 ± 0.4	1,216 ± 31
IBRS	Porcine	12 ± 0.2	141 ± 6	111 ± 0.8	1,768 ± 32
HeLa	Human	47 ± 7.8	2,401 ± 120	178 ± 4.1	661 ± 15
CV-1	Monkey	106 ± 0.8	2,371 ± 29	157 ± 4.0	1,845 ± 114
BSC-1	Monkey	97 ± 2.0	858 ± 77	114 ± 4.2	456 ± 20
BHK-21	Hamster	144 ± 0.2	2,489 ± 58	183 ± 23.8	967 ± 23
SIRC	Rabbit	25 ± 0.9	2,489 ± 225	59 ± 3.9	285 ± 9
RK-13	Rabbit	30 ± 1.6	978 ± 57	69 ± 2.3	263 ± 31
MDBK	Bovine	59 ± 0.3	84 ± 1	111 ± 5.6	140 ± 2

[a] Expressed as nmoles of ONPG hydrolyzed in 1 min at 37°C per 5×10^4 cells.
[b] AraC was used at a final concentration of 40 μg/ml.

with respect to the parental white plaques. Several SPV plaques positive for β-gal expression were picked and reisolated by consecutive rounds of plaque purification in the presence of X-gal. Four to five successive plaque purifications were necessary to dilute out parental virus not displaying the blue plaque phenotype. In subsequent plaque assays, exclusively blue plaques were detected (not shown), indicating that the recombinant SPV had the *LacZ* cassette stably inserted in the viral genome. One virus clone, SPV-LZ4, was selected for further characterization. Both the growth rate and the plaque size phenotype of SPV-LZ4 in ESK-4 cells were similar to those of the parental virus (not shown). The genomic structure of SPV-LZ4 DNA, analyzed by PCR using oligonucleotide primers external to the insertion site and flanks, confirmed that a DNA fragment of the correct length had been inserted into the intended location in the SPV genome (not shown).

In parallel, the same *LacZ* cassette from plasmid pUCLZ-7 was inserted in the genome of VV. A recombinant virus expressing β-gal, VV-LZ2, was isolated by plaque purification.

Expression of β-gal by SPV-LZ4 and VV-LZ2 in different cell lines

To initially characterize the host species restriction of SPV *in vitro,* we measured β-gal expression in cells infected with SPV-LZ4. Monolayers of 11 cell lines from six different mammalian species were infected with either VV-LZ2 or SPV-LZ4, in the presence or the absence of cytosine arabinoside (araC), an inhibitor of DNA replication. As expected from the broad host range of VV, enzyme activity was detected in a variety of VV-LZ2-infected cells, although to widely different levels (Table 1). The highest levels of β-gal activity were obtained in cell lines that are routinely used to grow VV-like HeLa, CV-1, or BHK-21 and also in the rabbit cell line SIRC. About 10-fold less β-gal activity was present in lysates from three of the porcine cell lines included in this study (ESK-4, ST, and IBRS). When DNA synthesis was inhibited by addition of araC, protein expression was significantly reduced, although to different levels in different cell lines. The observed effect of araC is consistent with the presence of both early and late promoter activities in $P_{E/L}$.

β-Gal expression in porcine cell lines was higher when they were infected with SPV-LZ4 than when infected with VV-LZ2. Significantly, SPV-LZ4 induced approximately 10-fold more expression of the reporter gene than VV-LZ2 in three of the four pig cell lines tested (ESK-4, ST, and IBRS). In addition, detectable levels of β-gal activity were also present in the lysates of SPV-LZ4-infected cells of nonporcine origin. Virus-induced expression of β-gal in CV-1 cells was comparable to that of infected swine cell lines, and significant expression levels were also present in BHK-21, HeLa, and BSC-1 cells. Nevertheless, it should be noted that SPV-LZ4 induced much less β-gal expression than VV-LZ2 in the cell lines of nonswine origin. As in the case of VV-LZ2-infected cells, addition of araC produced a reduction in the levels of β-gal in SPV-LZ4-infected cells, suggesting functional temporal promoter regulation of β-gal expression in those cells.

Bovine MDBK cells rendered the lowest levels of β-gal expression upon infection with either SPV or VV recombinant viruses. This result is not surprising, since this cell line was reported to be nonpermissive for SPV infection (Foley *et al.,* 1991; Garg and Meyer, 1972) and does not

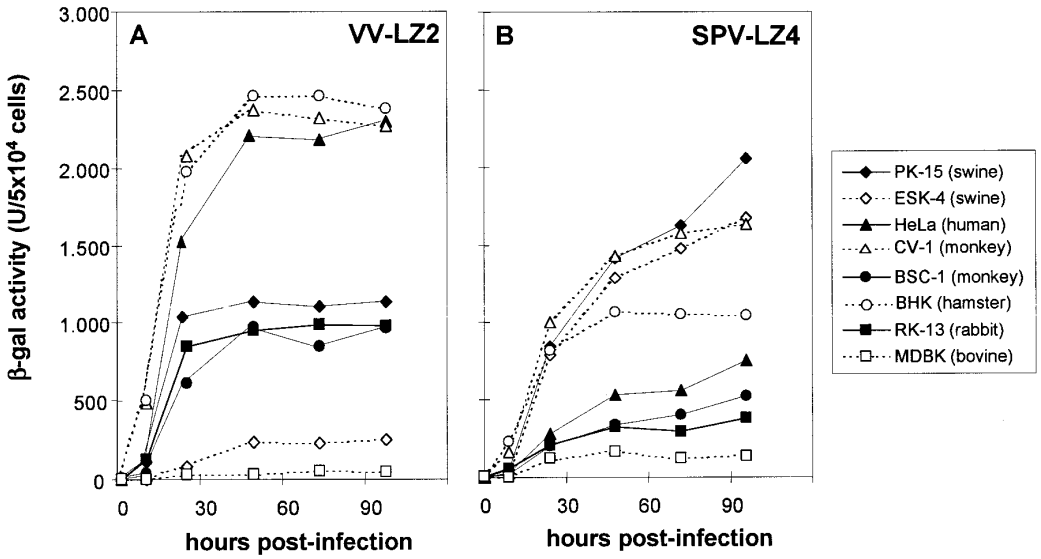


FIG. 3. Kinetics of β-gal expression. Cells infected with VV-LZ2 (A) or SPV-LZ4 (B) at an m.o.i. of 1 PFU per cell were lysed at different times postinfection and subsequently assayed for β-gal activity. Units of β-gal are expressed as nmoles of ONPG hydrolyzed in 1 min at 37°C per 5×10^4 cells. The various cell lines used are indicated on the right.

support expression of VV late gene products (Hruby *et al.,* 1980).

The kinetics of β-gal expression was followed by determining enzyme activity at different times postinfection (Fig. 3). In agreement with previous studies on the synthesis of SPV proteins (Massung and Moyer, 1991), β-gal expression in SPV-LZ4-infected cells was delayed with respect to those infected with VV-LZ2. Cells infected with VV-LZ2 reached maximum levels of β-gal activity by 48 h postinfection, whereas 72–96 h of infection was necessary in SPV-LZ4-infected cells.

Replication of SPV-LZ4 and VV-LZ2 in different cell lines

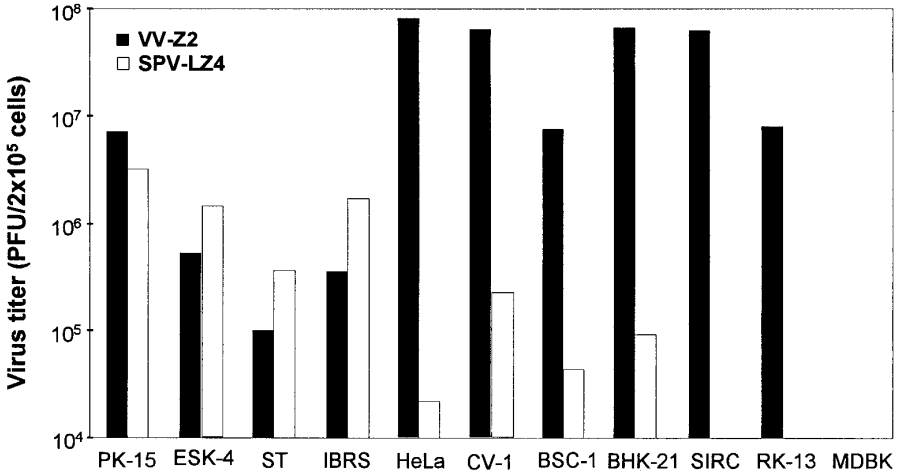
The observation that SPV induced gene expression in a variety of cell lines does not necessarily indicate that the virus can productively infect those cells, as exemplified by avipoxviruses or certain strains of VV, such as MVA, which are able to induce normal levels of gene expression and DNA replication in cells that do not produce infectious progeny (Paoletti, 1996; Somogyi *et al.,* 1993; Sutter and Moss, 1992). Since SPV is reportedly restricted to swine *in vivo,* it was of interest to determine whether SPV-LZ4 productively replicates in cell cultures from nonporcine origin. Thus, we tested the ability of SPV-LZ4 to grow in different cell lines and compared it to VV-LZ2 (Fig. 4A). The results obtained paralleled the gene expression data described above. As expected, recombinant VV was able to multiply in all the cell lines tested except in the nonpermissive MDBK cells. Similar to the β-gal activity assays, a high level of replication of VV-LZ2 occurred in HeLa, CV-1, BHK-21, and SIRC cells. A lower level of VV replication occurred in three of the porcine cell lines tested (ESK-4, ST, and IBRS). In contrast to VV, SPV-LZ4 replicated more efficiently in porcine cell lines than in the nonporcine cell lines. Moreover, the high efficiency of SPV-LZ4 replication in porcine cells resulted in SPV-LZ4 final virus yields being similar or even superior to VV-LZ2 yields in those cells.

Surprisingly, there were significant increases in virus titers of SPV-LZ4 in CV-1, BHK-21, BSC-1, and HeLa cultures over a 6-day infection period, indicating that SPV-LZ4 was actively replicating in those cells. No virus replication was apparent in MDBK and the rabbit cell lines SIRC and RK-13, since titers were lower than those of input virus. These results were not a particular feature of the recombinant SPV, since similar results were obtained when cell lines were infected with parental SPV (data not shown).

We also determined the ability of SPV-LZ4 to undergo serial passages in different cell lines. Six sequential passages were performed as described under Materials and Methods, and the virus titer was determined after each round (Fig. 4B). Again, the results obtained paralleled those of gene expression and viral replication experiments. Virus titers were maintained during passages in the two porcine cell lines, PK-15 and ESK-4, as well as in CV-1 and BHK-21, although viral titers in the latter remained consistently lower. These results confirm viral replication to different extents in several of the nonporcine cell lines tested and indicate that the virus is not readily adapted to nonpermissive cells.

Given that SPV is naturally restricted to pigs, the detection of a certain degree of virus growth in cell lines of nonporcine origin was unexpected. Virus yields in some cells were low, particularly in the case of BSC-1 and

A



B

Sequential passage of SPV-LZ4 in different cell lines

	SPV-LZ4 titer*							
Passage No.	PK-15	ESK-4	CV-1	BHK-21	BSC-1	HeLa	RK-13	MDBK
1	7.0	7.0	6.1	5.5	4.7	4.6	4.8	4.2
2	7.3	7.3	5.8	5.6	4.7	3.8	ND	ND
3	7.4	7.2	5.9	5.6	4.3	3.1	ND	ND
4	7.3	7.0	6.0	5.6	3.6	2.5	ND	ND
5	7.4	6.8	5.8	5.4	ND	ND	ND	ND
6	7.3	6.9	6.0	5.1	ND	ND	ND	ND

*Expressed as $\log_{10}$ p.f.u./ml. ND, not detectable (< 2.0).

FIG. 4. Virus production in different cell lines. (A) Virus production. Monolayers of the indicated cell lines were infected at a m.o.i. of 0.1 PFU per cell with VV-LZ2 or SPV-LZ4. After 3 days (for VV-LZ2-infected cells) or 6 days (for SPV-LZ4-infected cells) cells were harvested. Virus titers were subsequently determined by plaque assay on BSC-1 (for VV-LZ2) or ESK-4 (for SPV-LZ4) monolayers. Virus titers at 0 time (after the adsorption period) were in all cases under 1×10^4 PFU/2×10^5 cells. (B) Titers obtained after serial passages of SPV-LZ4. The initial culture was infected at a m.o.i. of 0.25 PFU per cell. In subsequent infections, one half of the material from the previous culture was used for infection. Titers were finally determined on ESK-4 monolayers.

HeLa cells, where it could not be formally excluded that the low viral titers recovered were residual input virus. Therefore, we carried out other experiments to confirm and further characterize the *in vitro* host range of SPV. We tested the ability of SPV-LZ4 to grow and spread in several cell monolayers by performing plaque assays staining with X-gal (Fig. 5). SPV-LZ4 formed large blue plaques in ESK-4 and CV-1 cells with roughly the same plaquing efficiency. Small or diffuse plaques formed in BSC-1, HeLa, and BHK-21 cell monolayers with a low plaquing efficiency. Since detectable plaques arise from multiple rounds of viral replication, plaque formation was an unequivocal indication of viral multiplication in these cell monolayers. No blue plaques were detected in RK-13, SIRC, and MDBK cell monolayers, in agreement with virus production results (Fig. 4A).

DISCUSSION

In this communication we describe the development of a cloning system for the generation of recombinant SPV by adapting procedures devised for the genetic manipulation of other poxviruses. This is the first full description of such a cloning system, although SPV recombinants have reportedly been obtained by inactivation of the thymidine kinase (TK) gene (van der Leek *et al.,* 1994). The conventional procedure for the isolation of recombinant poxviruses based on TK inactivation results in a severe attenuation of the virus (Buller *et al.,* 1985). Because the rare vaccine-associated risks are a major concern in vaccine development, attenuation is regarded as a desirable feature of vaccine vectors based on recombinant VV, and efforts are directed to develop highly attenuated strains with decreased virulence (Moss, 1996; Paoletti, 1996; Tartaglia *et al.,* 1992). However, in the case of SPV, the virus naturally exhibits a limited pathogenicity (Kasza and Griesemer, 1962; Meyer and Conroy, 1972; Tuboly *et al.,* 1993), and therefore further attenuation by gene disruption might not be desirable, since it could result in a loss of vaccine efficacy. With this in mind we developed a SPV cloning system such that insertion or

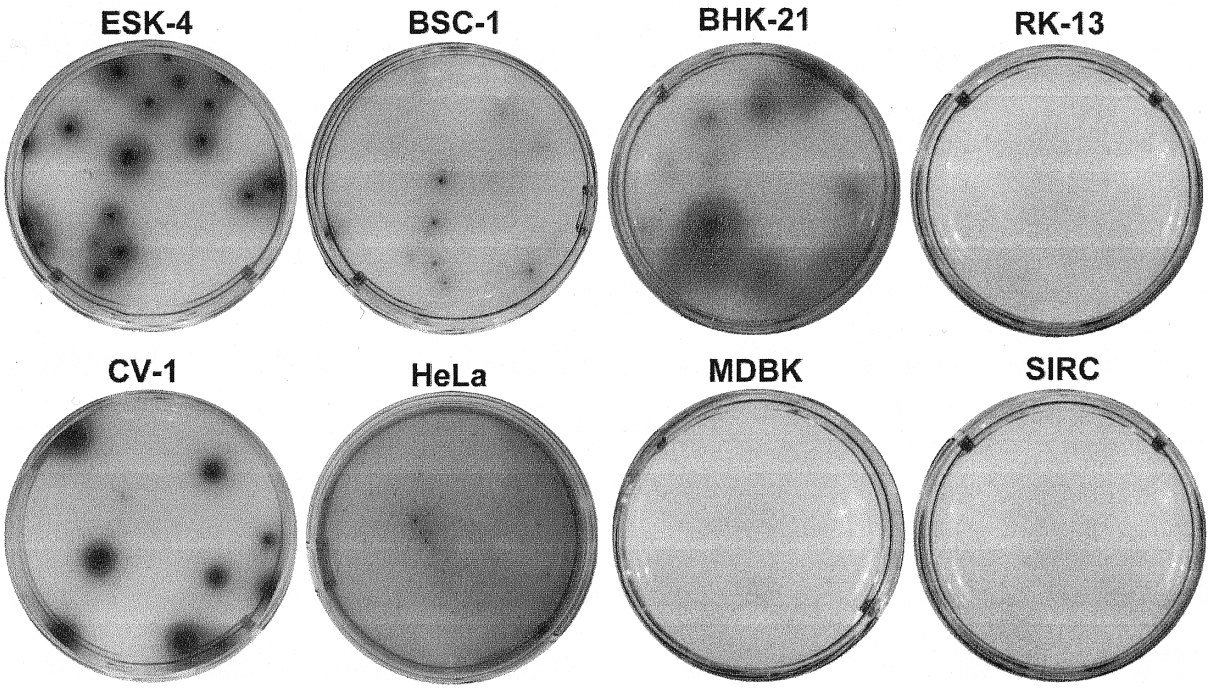


FIG. 5. Plaquing of SPV-LZ4 on different cell lines. Serial dilutions of a SPV-LZ4 crude virus stock were used to infect monolayers of the cell lines indicated. Virus dilutions used for each cell line were 10^{-6} for ESK-4 and CV-1, 10^{-5} for BSC-1 and BHK-21, and 10^{-4} for HeLa, MDBK, RK13, and SIRC. Plaquing efficiency, relative to that obtained on ESK-4 cells, was 0.73 for CV-1 cells, 0.15 for BHK-21 cells, 0.08 for BSC-1 cells, and 0.07 for Hela cells.

selection would not rely on the inactivation of any viral gene. If necessary, the system can be used in combination with defined modifications of the viral genome resulting in altered biological characteristics of the virus or enhanced immunogenicity of the foreign protein.

In addition to the insertion system, poxviral transcriptional regulators are required to ensure expression of the foreign gene. Since there is no available information concerning SPV promoters, we performed transient expression assays to evaluate promoter function of sequences derived from VV in a SPV context. Several facts led us to anticipate that VV promoters could be useful in directing high-level expression of genes. First, the limited sequence information from the SPV genome reveals that many VV regulatory features are conserved (Massung *et al.,* 1993). Second, the VV 7.5 early/late promoter has been shown to be functional in a SPV background, although no expression data are available (van der Leek *et al.,* 1994). Third, several studies have reported cross-recognition of promoter elements between different poxvirus genera (Boyle, 1992; Boyle and Coupar, 1986; Kumar and Boyle, 1990; Pearson *et al.,* 1991; Prideaux *et al.,* 1990). Our results demonstrate that strong VV promoters P11 and the synthetic promoter $P_{E/L}$ are functional in SPV-infected cells, indicating good cross-recognition of these VV promoters by SPV RNA polymerase.

The *LacZ* gene was chosen as a convenient marker gene for the characterization of the SPV cloning system because it provides a simple and reliable method for the detection of recombinant viruses (Chakrabarti *et al.,* 1985; Liu *et al.,* 1990; Panicali *et al.,* 1986; Spehner *et al.,* 1990). Furthermore, a number of studies have validated the β-gal assay for measuring promoter strength as well as for comparing expression levels induced by different recombinant poxvirus (Boyle, 1992; Davison and Moss, 1989a,b; Kumar and Boyle, 1990).

Recombinant SPV expressing β-gal offered new opportunities for analyzing SPV host range in cell culture. A number of studies have reported the host-restricted nature of SPV. Early studies reported that swine are the only host for SPV and that other species, including chick embryos, several laboratory animals, and humans, are not susceptible to the virus (Datt, 1964; Meyer and Conroy, 1972; Schwarte and Biester, 1941). Moreover, only porcine cell cultures have been reported to be permissive for SPV as no propagation of the virus has been detected in primary cell cultures (Kasza *et al.,* 1960; Mayr, 1959) or in established cell lines (Foley *et al.,* 1991; Garg and Meyer, 1972) derived from other species. Our results clearly show SPV gene expression, replication, and spread in certain nonporcine cell lines. The cumulative evidence presented in this study indicates that although SPV grows more efficiently in porcine-derived cell lines, the virus is able to propagate to some extent in most of the cell lines tested.

The observation that SPV can grow in a wide range of cell lines was surprising in light of the previous literature. Much of the information about SPV host range is derived from attempts to adapt SPV taken from lesions to cell cultures from different species. It is possible that these

previous studies were hampered by technical difficulties related to the slow and limited growth of the virus (Massung and Moyer, 1991), its lower level of replication compared with other poxviruses, and the lack of a reliable SPV plaque assay. Indeed, in our hands the level of replication in some cell lines was not enough to sustain repeated passaging in cell lines in which SPV replicates to a low level (Fig. 4B), and therefore failed attempts to grow SPV in cell cultures by blind passage should not be considered a bona fide indication of host restriction of the virus. It is unclear whether blind passages of SPV natural isolates performed in several studies represent in all cases an authentic adaptation of the virus or whether several passages are required to obtain enough virus to produce a generalized cythopathic effect. Interestingly, in one report, cythopatic effect was reported at the first passage of a natural isolate in PK15 and Vero cells, although repeated passaging in Vero cells could not be maintained (Paton *et al.,* 1990), suggesting that no adaptation was required for this natural isolate. In our experience, monolayer conditions and cell density can greatly influence the appearance and the yield of an infection (data not shown).

SPV replicated to very different levels in different cell lines. No evidence of SPV replication could be detected in bovine MDBK cells, a cell line which is also nonpermissive for VV (Hruby *et al.,* 1980). This result was in agreement with previous reports of failed adaptation of SPV to MDBK cells (Foley *et al.,* 1991; Garg and Meyer, 1972). Rabbit SIRC and RK-13 cells also failed to support detectable SPV replication. In this regard it is interesting to note that SPV apparently lacks the homolog of VV host range gene K1L (Massung *et al.,* 1993), which is required for VV replication in RK-13 cells (Perkus *et al.,* 1990).

Recombinant SPVs have been considered promising vaccines for pig diseases (Foley *et al.,* 1991; Tuboly *et al.,* 1993; van der Leek *et al.,* 1994). In this respect, a recombinant SPV expressing β-gal represents a new tool for characterizing and potentially improving its properties as a vaccine vector. Two important issues relevant to the use of poxviruses as vaccines are their efficacy and safety. Several findings presented here are pertinent to vaccine development. First, in general SPV was more efficient than VV in directing foreign gene expression in porcine cells. Thus, SPV may be a better vector for promoting high-level expression of immunogens in pigs, and indeed SPV appears more efficient than VV in infecting swine by different routes of inoculation (Datt, 1964). Second, SPV host restriction was not as narrow as anticipated, and this trait should be carefully considered in connection with vaccine safety issues. Similar to the orthopoxviruses, it is possible that one or several host range genes present in SPV modulate the ability of the virus to carry out gene expression in different cells. Therefore, one might be able to genetically curtail SPV host range by deleting SPV genes, to make more restricted vaccine vectors for swine.

It is also tempting to speculate that SPV tropism or replication efficiency could be extended by insertion of host range genes from other poxviruses, as is the case with ectromelia virus, whose host range can be altered by insertion of a host range gene from other orthopoxviruses (Chen *et al.,* 1992). SPV and VV are immunologically and genetically distinct (De Boer, 1975; Massung and Moyer, 1991; Ouchi *et al.,* 1992), and therefore it can potentially be used for heterologous boosting in combination with other poxviruses.

The reported *in vivo* host restriction of SPV to swine (Datt, 1964; Schwarte and Biester, 1941), its natural host, seems to be more strict that the *in vitro* host range of the virus. This is also the case with other host-restricted poxviruses, such as ectromelia and smallpox (Fenner *et al.,* 1989), which also display a wider host range in cell culture than in animals. Much work has been carried out in elucidating the viral determinants of host range in cell culture, and these differences indicate that additional determinants are acting *in vivo.* Obviously, genes involved in interactions of the virus with the immune defenses or spread within tissues are good candidates for modulating the *in vivo* host range. Awaiting more experimentation, the determinants of host restriction *in vivo* and *in vitro* remain an interesting problem in the biology of poxviruses.

MATERIALS AND METHODS

Viruses and cell lines

SPV (Kasza strain) was obtained from the American Type Culture Collection (ATCC). vRB12, a non-plaque-forming mutant of VV, WR strain (Blasco and Moss, 1991, 1992), was used as the parental virus from which a recombinant VV expressing β-gal (VV-LZ2) was constructed.

All the cell lines used in this study were obtained from the American Type Culture Collection. SPV was grown in PK-15 (porcine kidney) cells and VV in CV-1 (African green monkey kidney) cells. Plaque assays for SPV were carried out on ESK-4 (embryonic porcine kidney) cell monolayers and VV plaque assays were performed on BSC-1 (African green monkey kidney) cells. The following cell lines were also used: ST (porcine testis), IBRS (porcine kidney), HeLa (human cervix), BHK-21 (Syrian hamster kidney), RK-13 (rabbit kidney), SIRC (rabbit cornea), and MDBK (bovine kidney). All cell lines were grown in Eagle's minimum essential medium (EMEM) except ESK-4, ST, BHK-21, and MDBK cells, which were grown in Dulbecco's EMEM (DEMEM). Unless noted otherwise, all media contained 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. ESK-4, ST, BHK-21, and MDBK cells were supplemented with nonessential amino acids.

Virus growth

SPV stocks were prepared in PK-15 cells. The cultures were infected with plaque-purified virus and incubated at 37°C until complete cytopathic effect (CPE) was reached. Virus was liberated from cells by three successive cycles of freeze–thawing, sonicated briefly, and stored in aliquots at −70°C. SPV titers were determined by plaque formation on ESK-4 cell monolayers. Plaque assays were performed in liquid medium (6–7 days) or under a 0.6–1.0% low-melting-point agarose overlay (8–9 days), until visible plaques appeared after staining with crystal violet. For β-gal staining in plaque assays, a second agarose overlay containing 300 μg/ml of X-gal was added at 6 days postinfection, and blue plaques were visible after a further 6- to 12-h incubation at 37°C. VV mutant vRB12 used in this study was amplified by infecting cultures at high multiplicity of infection (m.o.i.), whereas growth and plaquing of recombinant VV-LZ2 were carried out following standard procedures (Earl and Moss, 1991).

SPV and VV host range *in vitro* were compared by infecting different cell monolayers containing 2×10^5 cells at a m.o.i. of approximately 0.1 PFU/cell for 1 h at 37°C. After the adsorption period, virus inocula were removed, and the cultures were then washed once with prewarmed DMEM and incubated with fresh medium (2.5% FBS in DMEM). Infections were arrested by freezing the cultures at time 0 (after the adsorption period), 3 days (for VV) or 6 days (for SPV) postinfection. Virus titers were subsequently determined by plaque assay on BSC-1 (for VV) or ESK-4 (for SPV) monolayers.

Enzymes and reagents

Restriction enzymes, T4 DNA ligase, and alkaline phosphatase from calf intestine were supplied by New England Biolabs or Boehringer-Mannheim and used as specified by the manufacturers. PCR reactions for the generation of DNA flanks of SPV genome were performed with Pfu DNA polymerase from Stratagene. The Expand Long Template PCR system from Boehringer was used in the PCR reactions to analyze the recombinant SPV genomic structure. Lipofectin reagent was supplied by Gibco-BRL. Purified *Escherichia coli* β-galactosidase (grade VIII) and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were purchased from Sigma, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) from Biomol.

Construction of plasmids

Figure 1 depicts the overall cloning scheme. Plasmid pUCLZ-7, containing the *LacZ* gene downstream of a VV early/late synthetic promoter, was constructed as follows: a DNA fragment containing the promoter sequence and several restriction sites was generated by annealing the following complementary oligonucleotides in which the overhanging cohesive ends are underlined: 5′-GATCCATCTCGAGTTATTTATATTCCAAAAAAAAAAAATAAAATTTCAATTTTTCCATGGGCTAGCAGATCTAAGCTT-3′ and 5′-GATCAAGCTTAGATCTGCTAGCCCATGGAAAAATTGAAATTTTATTTTTTTTTTTTGGAATATAAATAACTCGAGATG-3′. This DNA fragment was cloned into the *Bam*HI restriction site of plasmid pUC19 to generate pUCPTS-3. Cloning of the oligonucleotide recreates only one *Bam*HI site just downstream of the VV promoter, and therefore pUCPTS-3 contains a unique *Bam*HI site. A *Bam*HI fragment derived from plasmid pSC11 (Chakrabarti *et al.,* 1985), containing the *LacZ* gene, was then inserted into the *Bam*HI site of pUCPTS-3 to generate pUCLZ-7.

The β-gal insertion/expression vector, plasmid pGSPV-LZ4, was constructed by placing the *LacZ* cassette between SPV genomic sequences for homologous recombination. To generate the DNA flanks of SPV genome, two sequences from the intergenic region that separates ORFs C18L and C19L in the SPV *Hin*dIII C DNA fragment (Massung *et al.,* 1993) were amplified by PCR using primers 5′-CAAATATTCTAGATATTCAA-3′ (*Xba*I site underlined) and 5′-GTAAATAAGCTTCTCTTTTT-3′ (*Hin*dIII site underlined) for the creation of a 509-bp right flank, and primers 5′-AAAAGAGATCCTATTTACA-3′ (*Bam*HI site underlined) and 5′-AATATACTGAGCTCATACTA-3′ (*Sac*I site underlined) for the creation of a 486-bp left flank. After PCR amplification and digestion with the appropriate restriction enzymes, the flanking sequences were cloned sequentially into unique restriction sites in plasmid pGEM-7. Finally, the resulting plasmid, pGSPV, was digested with *Hin*dIII and ligated to a *Hin*dIII fragment containing the promoter-driven *LacZ* gene derived from pUCLZ-7.

Plasmid pRB20-LZ1, used to insert the *LacZ* gene into VV genome, was constructed from plasmid pRB20, a derivative of pRB19 (Blasco and Moss, 1995) in which an *Eco*RI fragment containing the gpt cassette had been removed (unpublished results). A *Hin*dIII fragment containing the *LacZ* cassette was isolated from plasmid pUCLZ-7 and ligated to *Hin*dIII-digested pRB20.

Isolation of SPV and VV recombinants

The procedure used to construct a SPV recombinant expressing β-gal (SPV-LZ4) was adapted from methods previously described for VV (Earl and Moss, 1991). Preconfluent monolayers of PK-15 cells grown in T-25 flasks were infected with SPV at a m.o.i. of about 0.2 PFU. The virus was allowed to adsorb for 1 h at 37°C and cells were transfected with 5 μg of calcium phosphate-precipitated plasmid DNA. Eighteen hours after infection, the transfection medium was removed and replaced with fresh EMEM–2.5% FBS, and cells were incubated at 37°C until complete CPE was reached (usually 6–7

days). Recombinant viruses expressing β-gal were isolated by plaque assay on monolayers of ESK-4 cells in the presence X-gal. Isolation of recombinant viruses was carried out by four to five sequential rounds of plaque purification. The genomic structure of recombinant viruses was analyzed by PCR, using primers derived from the SPV genomic sequence flanking the insertion site 5′-TGACCCTAAATATGTACCAGAAATAATACG-3′ (JB9) and 5′-TCGTAAATATGACATAAAACCATTATTTATTG-3′ (JB10). The PCR amplification of a 4.3-kb fragment, instead of the 11 fragment obtained from wild-type SPV, was indicative of a correct insertion of the *LacZ* gene.

A VV recombinant expressing the *LacZ* gene (VV-LZ2) was isolated using a selection method based on plaque formation (Blasco and Moss, 1995). Briefly, the system consists of two components: a mutant VV lacking the *vp37* gene, which has a nonplaquing phenotype, and a plasmid vector (pRB20) that, through homologous recombination, can simultaneously introduce a foreign gene (*LacZ* in this case) and a functional *vp37* gene that repairs the nonplaquing mutation in the VV genome.

β-Gal assays

In transient expression assays, monolayers of PK-15 cells grown on 24-multiwell trays were inoculated with wild-type virus (SPV or VV) at a m.o.i. of approximately 5 PFU/cell. The virus inoculum was removed after 1 h and cells were then transfected with various amounts of plasmid DNA, using lipofectin reagent as recommended by the manufacturer. After 5 h, transfection medium was removed and replaced with EMEM–2.5% FBS without phenol red, and cells were further incubated at 37°C for 96 h. At this time infection was arrested by freezing the cultures. Detection of β-gal activity was performed essentially as described by Davison and Moss (1989a,b). Infected cells in culture trays were frozen and thawed, scraped into the medium, and disrupted by adding 25 μl of chloroform and 25 μl of 0.05% sodium dodecyl sulfate. Portions of the lysates were incubated with Z buffer–ONPG solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 40 mM 2-Mercaptoethanol, 4 mg/ml ONPG) at room temperature. The yellow color which developed after 15–30 min was recorded as absorbance at 405 nm. β-Gal activity present in each sample was estimated with reference to a standard curve obtained with commercial *E. coli* β-gal, after subtracting the background values obtained with lysates from infected cells that had been transfected with pUC19 control plasmid.

Expression of β-gal by the recombinant viruses SPV-LZ4 and VV-LZ2 was assayed as described above with the following modifications. Monolayers of different cell lines grown on 96-multiwell trays were infected with the recombinant viruses at a m.o.i. of about 1 PFU/cell. After removing the inocula and washing the monolayer once, cells were incubated with EMEM–2.5% FBS without phenol red at 37°C in the presence or the absence of 40 μg/ml of araC. At the various times the cultures were frozen and cell lysates were assayed for β-gal activity directly in the 96-multiwell trays. Quantitation of β-gal present in the samples was performed as described above, after subtracting background values obtained with lysates from uninfected cells.

Serial passages of SPV

Different cell line monolayers containing 1×10^6 cells were inoculated at a m.o.i. of about 0.25 PFU/cell. After a 2-h adsorption period at 37°C, the inoculum was removed and the monolayers were washed once to remove unadsorbed virus. Fresh medium was added and the cultures were incubated at 37°C for 6 days. At this time, cells were removed from the plate by scraping and lysed by three successive cycles of freeze–thawing, followed by mild sonication. One half of each lysate was then used to inoculate a fresh monolayer of the same cell line. This procedure was repeated for six sequential passages. Finally, samples from each passage were assayed for the presence of infectious virus by plaque titration on ESK-4 monolayers.

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